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CONTRIBUTIONS TO THE BIOLOGY OF RHIZOBIA. V. THE ISOLATION AND CULTIVATION OF RHIZOBIA IN ARTIFICIAL MEDIA.

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IT is with a somewhat guilty conscience that this paper is submitted. It is directly prompted by the fact that numerous inquiries have been received as to details regarding the isolation and cultivation of the root nodule microbe of leguminous plants. While I am now well satisfied that rhizobia are very easily isolated and grown in artificial culture media, I am nevertheless only too conscious of my earlier repeated failures and mistakes, and the failures of others. These facts should have prompted me to publish the finally successful efforts as soon as possible in order to assist and simplify the work of others who were contemplating research along similar lines. In a recent article by GROSVENOR,[1] outlining some rhizobia culture work done by Dr. GEORGE T. MOORE of the Department of Agriculture, the statement is made that "after much labor he (MOORE) isolated the nitrogen-fixing bacteria," from which we are led to conjecture that even at this late date similar difficulties were encountered. With perhaps the one exception of Miss DAWSON, so far as can be ascertained, no investigator has published a detailed description of the methods of procedure in the isolation and cultivation of rhizobia. It is then with the view of correcting this neglect that this paper is submitted.

The rhizobia of the various leguminous plants examined, isolated, and cultivated by the writer, such as those of red clover, white clover, bur clover, sweet clover, garden peas, and alfalfa, gave practically the same results so far as methods were concerned. The early difficulties and failures were due entirely to ignorance with regard to the behavior of these organisms in artificial media, as has been set forth in previous papers. However, in spite of the readiness with which rhizobia are cultivated, the student must observe certain precautions in order that satisfactory results may be obtained. After numerous trials I have finally found the following methods to give the most satisfactory and most uniform results.

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[1] GROSVENOR, GILBERT H., Inoculating the ground. A remarkable discovery in scientific agriculture. *The Century Magazine* **68**:831–839 (October). 1904.

1. *Securing tubercle-bearing roots.*—Taking it for granted that it is desired to isolate and cultivate the rhizobium of some herbaceous leguminous plant, it is of considerable importance that the roots should be taken from plants growing in soil free from contamination with surface sewage, away from thickly populated areas, as vacant lots in large cities, etc. The object is to obtain tubercles which are comparatively free from foreign bacteria. Experiments with roots and nodules from plants growing in vacant lots in Chicago showed that they were covered externally with multitudes of microbes, which interfered very considerably with the rhizobia experiments.

Select healthy looking plants growing in loose soil, dig up the roots carefully; shake off the dirt very carefully, as the nodules are quite easily removed. By means of a sharp knife cut off root portions having well-developed single nodules or small groups (two or three) of nodules, and place them in a clean sterile container in which to carry them to the laboratory. Do not take roots with nodules that appear quite dark (bluish hue) at the base, as these are old tubercles and are quite badly contaminated with foreign soil microbes. Do not take roots with large tufts of nodules for similar reasons, and for the further reason that these nodule clusters are difficult to clean.

2. *Cleaning and garbling roots.*—At the laboratory look the material over carefully. Remove all foreign vegetable substances. Reject all roots or rootlets showing decay or discoloration. By means of the pocket knife cut away root portions which are undesirable. Now place this carefully garbled material under a faucet and let the water run over it to wash away dirt and sand. Care is necessary, as a strong flow or careless handling may remove the most desirable nodules. Remove all dirt, using a small brush, if necessary, to remove clinging sand and soil particles.

3. *Selecting nodules.*—After this cleansing, look the material over carefully. By means of a pair of small, blunt-pointed, clean, sterile tweezers pick off young, clean-looking, but well-developed nodules and drop them into a small, clean, sterile beaker about half full of thoroughly boiled water (boil this water yourself for half an hour).

About ten nodules should be selected. This is more than is required but allowance should be made for loss through accident.

4. *Brushing and washing nodules.*—Take up each tubercle by means of the blunt tweezers and clean it by means of a sterile camel's hair brush, rinse back and forth in the water, and then drop the cleansed nodules into a second small beaker half full of boiled water. Stir the tubercles about in this second beaker by means of a sterile glass rod or a small section lifter. By means of the section lifter transfer the nodules to a sterile test tube, about half full of boiled water. The camel's hair brush removes many microbes and sand and soil particles which cannot be removed in the following rinsing process.

5. *Rinsing the nodules in the test tube.*—Place the thumb over the mouth of the test tube and shake vigorously for five to ten seconds. Decant the water by holding a sterile wire gauze over the mouth of the test tube (after a little practice the water can be decanted without the use of the wire gauze). Add more water, shake, and decant as before. Repeat this process *ten* times. The object is still further to get rid of microbes clinging to the exterior of the nodules.

6. *Sterilizing the exterior of the nodules.*—After the last rinsing, described in the previous section, add to the test tube, in the place of water, a 5 per cent. carbolic acid or formalin solution and shake vigorously for eight seconds and decant the disinfectant immediately. The object is to kill microbes which may still be present upon the exterior and in the cork tissue and epidermal cells of the nodules. Naturally the antiseptic must be used quickly to prevent it from entering the interior of the nodule and killing the rhizobia themselves. Of these two disinfectants I am inclined to favor the carbolic acid, as it penetrates tissues less readily.

7. *Removing the disinfectant.*—This must be done at once. Rinse in boiled water (as in section 5) *five* times in rapid succession, to get rid of all traces of the disinfectant. The importance of this is evident, for should any considerable trace of the disinfectant remain it would destroy the rhizobia in the next process. No tests have been made to determine what the resisting power of rhizobia is to these disinfectants. I am inclined to believe that they have considerable resisting power, as is evidenced by their behavior in acid culture

media. Microbes differ widely in this regard. For example, typhoid fever germs and others, survive in a 0.5 per cent. carbolic acid solution.

8. *Crushing the nodules.*—Decant the last supply of water and transfer the tubercles to a thoroughly sterilized watch glass or similar small container (small salt cellar does well), and crush the tubercles with a sterile solid glass rod with rounded end. The crushed tubercles with the moisture that remained from the last rinsing form a pulpy mass. Cover with a sterile cover, such as a glass plate or a watch crystal, and set aside until ready to make the isolation cultures. If some time is required, the covered crush preparation should be placed upon a sterilized portion of the table and covered with a sterilized beaker or bell jar to present contamination by means of air microbes.

9. *Preparations for isolating rhizobia.*—It is assumed that the desired culture media have been prepared in the usual way. I would advise using the usual very slightly alkaline solid media of beef extract, salt, gelatin, and agar. Only sufficient agar should be used to give it solidity (about 1.5 per cent.). A number of test tubes about one-half to two-thirds full of the culture medium, sterilized by the fractional method, should be on hand ready for use. Six of these tubes should be placed in the steam sterilizer so that the medium may be thoroughty liquefied. This will require half an hour or more. They should be placed in the sterilizer about the time that the work of cleaning the tubercles is begun. Three thoroughly sterilized Petri dishes should be on hand ready for use. Number these one, two, and three.

10. *Preparing the test tubes for inoculation.*—When the culture medium in the test tubes is thoroughly liquefied, place the tubes into a beaker of moderately cool water. As soon as the water becomes hot from the heat of the tubes, replace it with fresh moderately cool water. While the tubes are still quite warm to the touch, place them in a beaker of water having a temperature of about 50° C., the object being to keep the medium liquefied. In this considerable care is necessary. The medium must not be too hot, nor should any portion of it be allowed to coagulate. The tubes should feel warm (not hot) to the touch when used. The tubes can be cooled

much more rapidly by holding them under a faucet of running water, but this is not recommended to the beginner because of the danger of cooling the tubes too much. Number four of the tubes and add two with blank labels for reserve purposes in case of accident or special requirement.

11. *Preparations for making the inoculations.*—A lighted Bunsen burner and a platinum wire loop fastened into a glass rod are all that is required. Wipe the table on which the work is to be done with a cloth moistened in some disinfecting solution.

12. *Inoculating tube no. 1; first attenuation.*—Heat the platinum loop in the Bunsen flame, allow it to cool and dip into the root nodule crush preparation, being careful not to include larger portions of tubercles, and introduce the loopful into test tube no. 1, shaking the platinum loop back and forth a few times within the medium. Replace the cotton plug in the tube, heat the loop in the Bunsen flame to a red glow, and place to one side. Now roll the test tube, kept in vertical position, between the hands to mix thoroughly the contents. Roll for five or six seconds.

13. *Inoculating tube no. 2; second attenuation.*—Take two loopfuls out of tube no. 1 and transfer them to tube no. 2; sterilize needle flames as before, and roll tube no. 2 as above.

14. *Inoculating tube no. 3; third attenuation.*—Take two or three loopfuls out of tube no. 2 and transfer to tube no. 3 and proceed as under section 12.

15. *Inoculating tube no. 4; fourth attenuation.*—Take three loopfuls out of tube no. 3 and transfer to tube no. 4 and proceed as under section 12.

Immediately after rolling, the tubes are to be replaced in the beaker of warm water to prevent the media from coagulating. Close application to detail is very important in order to avoid confusion. It is well to rehearse mentally the different manipulations before beginning with the tube inoculations or attenuations.

16. *Plating contents of inoculated tubes.*—Tube no. 1 may be rejected, but it is preferable to set it aside for control observations. Roughly estimated it contains several thousand rhizobia quite evenly distributed. Pour contents of tube no. 2 into Petri dish no. 1 which has been kept in a warm place (about 50° C.), spread the medium

evenly by inclining the dish slightly from side to side and set aside to cool. Pour contents of tube no. 3 into Petri dish no. 2, and contents of tube no. 4 into dish no. 3, spread medium and set aside. After the media in the Petri dishes are well coagulated, place the dishes where they will not be interfered with. They may be placed in an incubator provided the temperature is kept well below the melting point of the culture medium. I have obtained the most uniform results by keeping the plate cultures at the normal temperature of the laboratory.

About the third day the growths will begin to appear as very small light grayish specks; several hundred, more or less, in dish no. 1; twenty-five to thirty in dish no. 2; and perhaps only five to ten in dish no. 3. These are rhizobia cultures, and if the work was well done there will in all probability be no foreign microbes present. The cultures on the upper surface of the medium will be circular in outline, while those within the medium will be spindle-shaped. Growth of cultures is comparatively slow, thus giving ample time for study and to make tube cultures, plate cultures, etc. Tube cultures have been quite fully described in previous papers, likewise the morphology of the rhizobia grown in various artificial media.

Various other methods of isolating rhizobia have been tried successfully, but the method just described is recommended as giving the best results. It is somewhat lengthy in detail, but nevertheless simple in operation. It will be found that the isolation and cultivation of rhizobia is indeed simple, and even the beginner in the study of bacteriology will wonder what might have been the cause of the difficulties encountered by the earlier (1886 and later) investigators of the root nodule organisms. The difficulties however will become somewhat evident on examining the cultures microscopically. The morphological characteristics are found to be entirely changed, so that there is no similarity between the organism as it appears in the nodule and the organism as it appears in the artificial culture media.

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